IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: McDonald *et al.*. Group Art Unit: 1646

Serial No.: 09/360,242 Examiner: Landsman, R.

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For: *METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS*

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Sir:

I, JOHN R. McDONALD, declare as follows:

1. I am an inventor of and am familiar with the subject matter of the above-captioned application; and I have read the Office Action, mailed April 9, 2002, in connection with the above-captioned application.

2. I received B.Sc. and Ph.D. degrees at Napier College, Edinburgh, completed successful postdoctoral appointments in Canada and The United States before leaving academia for the biotechnology industry (Boulder CO, and San Diego CA). I have been involved in all aspects of the Research and Development process from project planning through IND filing. My research has focused upon growth factor signal transduction, multiple sclerosis, and the purification and characterization of neurotrophic factors and growth factor-mitotoxin fusion proteins. I have received several peer-reviewed awards and grants, including a US National Institutes of Health Small Business Innovation Research Grant. I am co-author of over fifty publications, and a named inventor on nine patent applications.

3. I am a founder of Osprey Pharmaceuticals Limited, Canada, and I am currently Vice-President Research & Development and a Director at the company. The conjugates described and claimed in the above-captioned application are broad-based, widely applicable anti-immunoinflammatory drugs for treatments, including treatment of secondary tissue damage-associated disorders including those that accompany central nervous system trauma and disease, including spinal cord injury, head injury, multiple sclerosis, amongst others, and for other inflammation-driven diseases as divergent as asthma, arthritis, HIV and cancer.

3. Tissue culture data obtained on the chemokine-toxins, OPL98110 and OPL98111 are of record in the Response and Declaration, dated 9/11/00 and in the subsequent published IDrugs article (2001, **4**(4): 427-442) by McDonald *et al.*, which is attached hereto and is incorporated into this DECLARATION; this data and additional data is provided below.

4. In my capacity as a Director, I have directed experiments described below, which demonstrate the effectiveness of conjugates of chemokine receptor-targeting agents for treatment of diseases that are characterized or caused by a pathophysiological inflammatory response. These conjugates provide a more selective and targeted delivery than previous ligand-directed delivery conjugates. The following exemplary results evidence these properties. Furthermore, as discussed below, *in vivo* effectiveness of the class of conjugates claimed in the application can be inferred from the data provided, the application and the knowledge of those of skill in the art in light of this application. The following discussion is organized into the following sections:

A. Exemplary experiments and results

B. Evidence for a nexus between chemokine-toxin *in vitro* data and *in vivo* data

C. Discussion and evidence that the chemokine superfamily of ligands and receptors are structurally and functionally distinct from those of the

cytokine family.

D. The teachings of Volk *et al.* and also the efficacy of different immune cell-depleting agents

A. Exemplary experiments and results

1. Materials and Methods

Materials and methods used in the experiments described herein are also set forth in the application.

a. Construction of Genes

Conjugates were constructed as described in the above-captioned application. As described above, conjugates with a variety of specificities were constructed. Exemplary of these constructs are OPL98110 (an MCP-1-Shiga toxin conjugate), OPL98111 (an SDF-1β-shiga toxin conjugate), and OPL98112 (an eotaxin-shiga toxin conjugate).

b. Tissue culture protocols

1) Primary cultures

Protocols for adult human brain cell culture are performed as described in detail by Yong *et al.* ((1997) Culture of glial cells from human brain biopsies. In *Protocols for Neural cell Culture* (A. Richardson and S. Fedoroff, eds), Humana Press, St. Louis 157-172). Briefly, surgically resected brain tissue is cut into 1 mm cubes and incubated in 0.25% trypsin for one-hour at 37 °C. The suspension is passed through a 130 μm nylon filter which dissociates the tissue into single cells. Following centrifugation (15,000 rpm, 25 min.) in 30% Percoll, the supernatant contains viable neurons while the pellet is comprised of tissue debris, myelin, and red blood cells. The neural cells are collected and plated onto uncoated tissue culture plastic. The cultures are incubated for 24 hours at 37 °C by which time the microglia adhere to the plastic while the oligodendrocytes remain in solution. Oligodendrocytes are decanted, centrifuged, and plated onto poly-L-lysine, to which

they adhere. Neurons and astrocytes do not survive this isolation process, however, the resulting populations of oligodendroglia and microglia are greater than 95% pure.

Neurons and astrocytes are derived from fetal brain specimens. Brain tissue is cut into small cubes and incubated with 0.25% trypsin and 100 μg/mg DNAase at 37 °C, as described in Oh *et al.* ((1996) *Glia 17*:237-53). The suspension is passed through a 130 um nylon filter and the filtrate is collected, washed, and seeded onto poly-L-lysine-coated tissue culture plastic to allow the cells to adhere. A Percoll centrifugation step is not required since most fetal axonal tracts are not myelinated. To purify the neuronal population the mixed culture is treated with 25 uM cytosine arabinoside (Sigma, St. Louis) which destroys the mitotically active astrocytes. To purify the astrocytic population the mixed culture is passaged in the presence of 0.25% trypsin, which kills neurons. Adult astrocytes are isolated in a similar manner.

In general, neural cell cultures are fed twice weekly with minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 20 μg/ml gentamicin, and 0.1% dextrose (Gibco, Grand Island, N. Y.).

Human peripheral blood leukocytes are harvested according to published methods (Chabot *et al.* (1997) *J. Clin. Invest. 100*:605-612, attached hereto). Briefly, venous blood is layered on to Ficoll-Hypaque (Pharmacia) and centrifuged for 30 min at 2500 rpm. The mononuclear cell fraction is collected, washed twice, and seeded onto uncoated tissue culture substrates. Two hours later, floating cells (mostly T lymphocytes) are removed to leave behind an adherent population that consists primarily of monocytes. These cells are used immediately in cytotoxicity experiments. Test cells are supplied with fresh medium containing control and test substances (at different concentrations) and incubated for a specified period (24-36 h). Cytotoxicity is then measured as the ability of adherent cells to reduce the vital dye MTT (see, *e.g.*, Mosmann (1983) *J Immunol Methods* 65, 55-63 and Gieni *et al.* (1995) *J Immunol Methods* 187, 85-93). In general, all hemapoietic cells (primary

cells, or the cell lines described below) are maintained in RPMI medium supplemented with 10% fetal bovine serum, 20 mg/ml Gentamicin and 0.1% dextrose (Gibco).

2) Cell lines

Cell lines derived from human mononuclear phagocytes are cultured using routine procedures. For example, monocyte-derived U937 and THP-1 cells, and the microglia-like CHME line from fetal brain (obtained from Dr. Tardieu, France). Human microglial cell lines established after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci Lett* 195, 105-8), have been used to test conjugates prepared as described in the above-captioned application. Numerous cell lines, including those of astrocytic and neuronal lineage, can be readily obtained from the ATCC (Rockville, MD) and successfully cultured using the instructions that accompany the shipment.

c. Immunohistochemistry

Indirect immunohistochemistry is routinely performed to confirm the purity of enriched cultures, and by extension, to distinguish between different cell types in a mixed culture. There are a variety of academic and commercially available cell type-specific antibodies that can be used to facilitate this process. Examples include, an anti-galactocerebroside (GalC) antibody to identify oligodendrocytes, an anti-glial fibrillary acidic protein (GFAP) antibody for astrocytes, an anti-Mac-1 antibody for microglia, and an anti-neurofilament antibody for neurons (anti-NFL).

In brief, live cells on cover slips are treated with an appropriate fixative (e.g., 4% paraformaldehyde for galactocerebroside, and 95% ethanol/5% glacial acetic acid, v/v). A predetermined concentration of the primary antibody is applied followed by an appropriate secondary antibody (typically, rhodamine or fluorescein-conjugated goat anti-rabbit or anti-mouse IgG). The stained cells are examined using a microscope equipped to detect immunofluorescence. Analysis of adherent cell cultures primarily relies upon indirect immunohistochemical staining and labeling, and

double labeling methods. Each cell type is counted in a sufficiently large number of randomly chosen microscope fields and the data are subjected to appropriate statistical analysis. Depending upon the mode and/or level of toxicity, that is to say, apoptosis versus necrosis and/or subtle versus gross toxicity, the degree of cell death is recorded either qualitatively (toxicity grade of 0 to 4 (see, *e.g.*,Noble *et al.* (1994) *Brain Res* 633, 83-90) or quantitatively (the number of dead cells as a percentage of the total population (see, *e.g.*, Oh *et al.* (1996) *Glia 17*:237-53). The promoting effects of bFGF and astrocyte extracellular matrix on process outgrowth by adult human oligodendrocytes are mediated by protein kinase C (*Brain Res* 757, 236-44). In most instances data are analyzed using a one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons. Suspended cells are analyzed using a flow cytometer (see, *e.g.*, Williams *et al.* (1997) *Brain Res* 754, 171-80; Stuve *et al.* (1996) *Ann Neurol* 40, 853-63; Stuve *et al.* (1997) *J Neuroimmunol* 80, 38-46; Williams *et al.* (1992) *J Neuropathol Exp Neurol* 51, 538-49), which automates both data collection and appropriate statistical analysis (*e.g.*, equipment from Becton Dickinson).

d. Cytotoxicity Assays

Briefly, test cells are supplied with fresh medium containing control and test substances (at different concentrations) and incubated for a specified period (24-36h). Cytotoxicity is then measured as the ability of adherent cells to reduce the vital dye MTT (see, *e.g.*, Mosmann (1983) *J Immunol Methods* 65, 55-63 and Gieni *et al.* (1995) *J Immunol Methods* 187, 85-93). Cytotoxicity in suspended cell cultures is measured using a Coulter counter, where the absolute number of cells is taken as an index of the number of surviving cells per test condition. Finally, general cell survival and morphology are monitored throughout the experiments using phase inverted microscopy and exclusion of the dye trypan blue (Yong *et al.* (1997) Culture of glial cells from human brain biopsies. In *Protocols for Neural cell Culture* (A. Richardson and S. Fedoroff, eds), Humana Press, St. Louis 157-172).

e. Chemotactic Assays

The chemotactic effect of each conjugate is measured as a test of the biological activity of the ligand component. Several chemotactic assays are described in the literature and employed (see, *e.g.*, Stuve *et al.* (1996) *Ann Neurol* 40, 853-63; Stuve *et al.* (1997) *J Neuroimmunol* 80, 38-46). In brief, the top and bottom compartments of a modified Boyden chamber are separated by a 3 μm membrane coated with fibronectin. Hematopoietic responder cells, appropriate to the chemokine being tested, are placed into the top compartment of the chamber while test materials are placed in the bottom. After an appropriate period of time, the number of cells that have migrated in response to a chemotactic stimulus is recorded. Migrating T-lymphocytes fall off the membrane into the lower chamber and the can be counted using a Coulter counter. In contrast migrating MNPs are retained on the underside of the membrane, and consequently, the upper surface must be washed and the lower surface fixed, prior to staining with Coomassie Blue and analysis by light microscopy.

2. Results and discussion

As demonstrated in experiments described below, and in the previous DECLARATION, conjugates provided in this application target activated cells that express chemokine receptors; they do not target cells that do not express chemokine receptors. The following experiments demonstrate that chemokine receptor targeting conjugates target cells that express receptors to which the particular chemokine targeting agent binds and are cytoxic to such cells. These compounds distinguish between activated and quiescent target cells. It is the former that are associated with inflammatory damage. Furthermore, the results show a correlation between *in vitro* data and *in vivo* effectiveness.

a. ***In vitro*** **activity of OPL98110.**

OPL98110, which is a conjugate containing MCP-1 and shiga toxin, was prepared as described in the application. MCP-1 specifically binds to CCR2 receptors, which are present in the activated microglial cells in the CNS and on monocytes, macrophages and certain populations of T-cells..

Given its profile of cell and receptor selectivity OPL98110 (MCP-1, CCR2) is an appropriate chemokine-toxin conjugate for use in the nervous system. Our results demonstrated that it targets cells of monocytic lineage including THP-1 leukemia cells, primary human peripheral blood mononuclear cells (PBMCs) and T-cells. There was no evidence of an effect on primary human neurons or U251 cells (a glioma of astrocytic lineage). This chimera is effective on non-confluent target cells within 24 hours at doses as low as 1 μg/ml and will kill up to 70% of the culture at doses between 5 and 10 μg/ml. Entire cultures are eradicated within 48 hours. In specific experiments Human peripheral blood monocytes (from healthy donors) and THP-1 cells (a human monocytic cell line) were treated with 1:10 and 1:50 dilutions of Control B and OPL98110. Twenty-four hours later the cells were examined by phase contrast microscopy and representative fields were photographed and counted. OPL98110 caused marked membrane disruption and vacuolization in both cell types. Most of the treated cells appeared abnormal, and an increased amount of cellular debris indicated that some were already dead. At the lower concentration of the conjugate (1:50) 20-25% of both cell types were affected.

Note: Control A is tissue culture medium. Control B is a wash fraction obtained prior to the elution of the chemokine-toxin from the nickel-affinity resin. This fraction was heavily enriched in *E. coli* proteins. Unless otherwise indicated all procedures were carried out in triplicate. It appears that OPL98110 kills activated monocytoid cells in the short term ignoring the quiescent cells, which do not express the targeted receptor. As the quiescent cells become activated in culture (perhaps in response to released mediators from dead or dying cells) during the incubation

period, the appropriate receptors are expressed and they too become targets.

OPL98110 Activity On Non-Proliferating and Proliferating Target Cells

Several experiments showed that when THP-1 cells were grown to confluence and treated with OPL98110 (1 to 10 μg/ml) no cytotoxic activity was observed. Numerous studies, however, have shown that the cells will die upon treatment if the cells are seeded in tissue culture wells at non-confluent densities. For example, in one set of experiments, THP-cells were grown for 48 hrs in the presence and absence of OPL98110 (1:10 dilution) and cell viability examined by either microscopy or the ability to exclude trypan blue. Cells that exclude the stain are alive while stained cells are dead. Since THP-1 cells are naturally non adherent, and in order to produce a more accurate count, control and treated cells were dissociated from cellular debris by gentle pipetting prior to counting. After 48 hours, 7.4 ± 3% of the control cells were dead (i.e. stained) in comparison to 58.8 ± 13% of the OPL98110 treated group. This is a 51.4% difference. Sister wells examined after 96 hours revealed that control cells had proliferated and continued to appear quite normal and healthy while the chemokine-toxin treated cultures contained a lot of cellular debris, but few if any live cells. These cultures were split and allowed to incubate for a further seven days. Control THP-1 cells continued to thrive and proliferate. There were no surviving cells in wells split from OPL98110 treated cultures). These studies demonstrate that treated cells become sick, and eventually die, over an extended period of time, suggesting an apoptotic mechanism.

Conclusion

OPL98110 does not kill non-proliferating THP-1 cells because they are essentially quiescent have no room to migrate or proliferate and do not express the target receptors. It is only when they become activate and proliferate that they become susceptible to OPL98110. This is consistent with the upregulation of chemokine receptor on activated cells. Experiments on migrating cell were

performed to further establish these points. This is illustrated in Figure 2 of the attached article McDonald *et al.* (2001) *IDrugs 4*:427-442.

To establish that OPL98110 distinguishes between activated and quiescent cells carefully designed migration experiments were set up using THP-1 cells. In brief, the *in vitro* migration of activated leukocytes can be induced by chemokines and measured by counting cells that migrate through a 3 μm filter separating the top and bottom chambers of a modified Boyden tissue culture dish. Migration is usually complete in 2 to 3 hours but not every chemokine is an effective chemoattractant even if the cell has the appropriate receptor. For example MCP-3 is a THP-1 chemoattractant but MCP-1 (and hence, OPL98110) is not. Furthermore, a certain percentage of THP-1 cells are constitutively active and will migrate without any specific exogenous stimulus, to a region of low cell density. With suitably long incubation periods, however, it is possible to measure the effects of OPL98110 on the active cells that migrate to the bottom of a Boyden dish.

In our experiments, THP-1 cells were plated into the top chamber of modified Boyden dishes and the lower chambers contained culture medium with and without OPL98110. The cells on both sides of the filter were exposed to the chemokine-toxin. After 24 hours the cells in the top and bottom chambers were counted using a Coulter counter. There was no difference in cell numbers in the top chambers between control and tests indicating that equal numbers of cells had migrated under all conditions. In comparison to control, cell numbers in the bottom chambers of treated cells decreased as the concentration of OPL98110 increased. The chemokine-toxin conjugate induced a dose dependent (between 0.5 and 5 μg/ml) decrease in the number of cells in the bottom chamber.

Only the activated (migrating) cells were affected by the chemokine-toxin. For example after 24 hours, approximately 75-80% of stationary THP-1 cells treated with OPL98110 (1:50 dilution) appeared healthy when viewed under the microscope. The mean cell survival rate in migration assays using the same dilution

of the chemokine-toxin was 50 ± 15% (mean of 3 experiments in triplicate).

The over-expression of MCP-1 and target receptors have been observed in a wide range of cancers. For, example the chemokine is responsible for the large leukocyte infiltrates seen in breast, lung and ovarian cancers. MCP-1 has been shown to play a direct role in tumor associated angiogenesis (a first for an *α*-chemokine family member) and tumor progression. Consistent with this OPL98110 was found to be highly toxic to MCF-7 breast carcinoma cells in culture.

OPL98110 Activity On Non Target Cells

OPL98110 was tested on non-target, primary human fetal neurons and a human U251 glioma (astrocytic tumor) cell line. Neurons were activated with TNF-alpha to simulate inflammation. The glioma cells were aggressively proliferating, and hence, activated. Following a 24 hour exposure to OPL98110 (1:50 dilution) there was no detectable effect on either cell type. Immunohistochemical staining of the neurons for *β*-tubulin and the detection of apoptosis (TUNEL) revealed healthy, intact cells.

b. Activity of OPL98111

In some forms of inflammation a broad approach afforded by a conjugate, such as OPL98111 (SDF1-*β*- shiga toxin conjugate), is acceptable. It is acceptable, for example, for targeting activated cells in cancer. The *α*-chemokine SDF-1*β* only binds to CXCR4 receptors; this receptor subtype is found on a very wide array of cell types, in addition to leukocytes. As noted in the attached article McDonald *et al.* (2001) *IDrugs 4*:427-442 and the prior DECLARATION of record, the chemokine-toxin conjugate OPL98111 targets U251 (astrocytoma), HT-29 (human colon carcinoma), and THP-1 (monocytoid leukemia) cells in culture as well as primary human monocytes T-cells, and primary human neurons. We have also demonstrated that human foreskin fibroblasts and primary fetal astrocytes do not respond to OPL98111.

***In vitro* activity of OPL-98111**

We have found in our studies that the chemokine-toxin OPL-98111 (SDF-1β) targets U251 (astrocytoma), HT-29 (human colon carcinoma), and THP-1 (monocytoid leukemia) cells in culture (Figure 1, attached hereto), as well as primary human monocytes, T-cells, and primary human neurons. Figure 1 shows the cytotoxic activity of OPL-98111 on target cancer cells in culture.

It was observed that under comparable tissue culture conditions, only 60% of primary monocytes were killed by OPL-98111 at a dose (10 μg/ml) that was shown to eradicate most of the tested cancer cells (see, Figure 3 in the McDonald *et al.* (2001) *IDrugs 4*:427-442, which is attached). This indicates that only the activated population of isolated monocytes (*i.e*, those with upregulated CXCR4 expression) are targeted.

***In vivo* activity of OPL-98111 Xenograft model**

Unlike OPL98110, which kills cultured target cells in 12-24 hours, OPL98111 has lytic properties, at least at high concentrations (20 μg/ml lyses a typical target culture in less than five minutes). The effects of this agent on HT-29 human colon carcinoma cells were investigated *in vivo*.

In this xenograft model, tumors were seeded by injecting 1 million live HT-29 cells into the right flank of female SCID/CB17 Fox Chase mice and allowed to grow for up to 15 days prior to treatment. This protocol produces aggressive and heterogeneously sized tumors that make it difficult to measure the changes between control and treated animals (five intratumoral injections of microgram quantities over 5 days). Despite these shortcomings, OPL-98111 retarded tumor growth relative to control animals ($n = 4$ animals per group, $p < 0.0001$, by one-and two-tailed Student's t-tests and two-factor analysis of variance, ANOVA). In a second experiment, the effect of OPL-98111 on smaller tumors was more readily apparent ($n = 3$ animals per group, $p < 0.0001$, Student's t-test). Furthermore, histological examination of similarly sized tumors from treated and control animals revealed that treated tumors contained more dead and dying cells and less evidence of a blood

supply (Figure 2 attached hereto; see, also Figure 4 in McDonald *et al.* (2001) *IDrugs* *4*:427-442). The Figure shows the effect of OPL-98111 on xenografted HT-29 human colon carcinomas from SCID mice. It can be seen in the Figure that tumors from treated animals exhibited far less live tumor mass than untreated animals. In addition, the tumors in the untreated animals showed greater vascularization. Also, in the treated animals, the tumors contained abundant monocytic cells, which clear cellular debris.

In preliminary toxicology testing, OPL-98111 (5 mg/kg ip) had no apparent effect on normal mice whereas the same dose, administered intravenously, was lethal within 12 to 24 h. This dose far exceeds a therapeutic dose (approximately 20 to 200 μg/kg) of a typical ligand-toxin given systemically. In the 30 plus days that each xenograft experiment took to complete, no animals died and post-mortem histology did not reveal any detectable abnormalities in major organs. Thus, as discussed in the previous DECLARATION, toxicology testing indicated that the SDF-1β conjugate, despite the wide distribution of CXCR4 receptors, is less systemically toxic than initially thought.

The subcutaneous dose of 5 mg/kg OPL98111 had no apparent effect on normal mice; whereas the same dose given intravenously was lethal within 12-24 hours. A therapeutic dose, given systemically, of a typical ligand-toxin, would generally not exceed 0.25 mg/kg. In the thirty plus days that each xenograft experiment took to complete no animals died and post-mortem histology did not reveal any detectable abnormalities in major organs. Furthermore, histological examination of similarly sized tumors from treated and control animals revealed that treated tumors contained more dead and dying cells (63 v 32 %) and less evidence of a blood supply (no evident vascularization).

The results demonstrated that concerns about broad-acting chemokine-toxins in general, and OPL98111 in particular, appeared to be exaggerated given the tight association between chemokine receptor activation and upregulation in

inflammation, and the receptor down regulation that occurs on exposure to the ligand. It appears that these conjugates will be effective at relatively low concentrations and have a somewhat self-limiting effect.

c. *In vitro* and *in vivo* activity of OPL98112

No *in vivo* toxicity

OPL98112, a fusion of eoxtaxin and shiga toxin described in the application, was injected i.p. into normal mice every second day for 7 doses (14 mg/kg cumulative dose). On day 20 the mice were still well. No untoward organ damage was noted (examination of hole organs (lung, liver, brain kidney and gut).

In vitro activity

In vitro activity was tested on mononuclear cells, prepared as described above. Two separate samples of 50 % pure OPL98112 were tested on the monocytes, indicating that it is toxic to monocytes. The results are as follows:

Protein concen-tration μg/ml	Sample 1 % Cell Death of controls	Sample 2 % Cell Death of controls
19	81	81
9.5	41	46
3.8	32	25
1.9	25	25

Also, microscopic observations of OPL98112 on mononuclear cells indicate that it was more potent against monocytes (immature macrophages) than mature macrophages. Even at that relatively high concentration, approximately 19% of teh the primary cells are not killed. These appear to be a quiescent population that does not express the targeted receptr CCR3.

Summary and conclusions

To summarize, the three chemokine-toxins for which data is provided in this and the prior DECLARATION were designed to target different cells based upon the chemokine receptor targeting agent specificity. As shown, they exhibit the appropriate cell profiles. The conjugates do not affect cells that are known not to

express the target chemokine receptor(s) and, nor, as shown for OPL98110, non-proliferating and non-migrating (i.e., non-activated) target cells that are not yet expressing the relevant receptor.

With this knowledge and the published observations that specific chemokines are expressed in specific diseased cells (and not normal cells), the application describes the eradication of certain cell types *in vivo*. The planned Xenograft mouse models and the unresponsiveness of normal cells in these experiments demonstrate this. Thus there is evidence for a nexus between *ex vivo* and *in vivo* observations and is described in detail below.

As shown above, in DECLARATION of record and in the attached article, McDonald *et al.* (2001) *IDrugs 4*:427-442, *in vivo* data shows that in two separate experiments growth of the tumors in animals treated with OPL98111 was retarded relative to control animals. Thus the molecule clearly eradicated targeted tumor cells *in vivo* (see, Fig. 2). Histological examination of similarly sized tumors from treated and control animals revealed that treated tumors contained more dead and dying cells (63 v 32 %) and less evidence of a blood supply (no evident vascularization). It is well established that in any Xenograft model the growing tumors become vascularized and receive a blood supply from the host animal. This involves the activation, proliferation and migration of endothelial cells and immune cells into the tumor and surrounding tissue. Hence, the conjugates, as claimed in this application, target and inhibit the activation, proliferation and migration of endothelial cells.

It is well established in the literature that the CXCR4 receptors targeted by OPL98111 are expressed on activated endothelial and immune cells. The application teaches that, based upon the profile of these receptors and the receptor specificity of SDF-1β (the chemokine receptor targeting agent in OPL98111), **activated**, **proliferating** and **migrating** endothelial and monocytic cells (i.e., monocytes and macrophages) should be eradicated. Upon examination of stained tissues from untreated animals, numerous small white circles representing the end of sectioned

blood vessels (Fig. 2) can be seen throughout the tumor tissue section. These circles represent the well-established angiogenic (neovascularization or new blood vessel formation) activity associated with growing tumors and immune cell infiltrates. In contrast, there was no evidence of any sectioned blood vessels in the remaining tumor areas of the tissue harvested from OPL98111 treated animals. Thus, OPL98111 exhibits an anti-angiogenic effect by eradicating activated, proliferating and migrating endothelial cells.

In addition, microscopic examination of the stained tissues reveals the presence of monocytic infiltrates in the small areas of tissue naturally undergoing necrosis. The mature macrophages are responsible for phagocytosing cellular debris and cleaning up the normal activity of these cells. Monocytes represent a population of immature cells that proliferate and differentiate into macrophages (since SCID mice were used, no T-cells are present). In tissues treated with OPL98111 and harvested immediately for examination, there was a stark lack of monocytic cells in necrotic regions. When treated tissues were harvested after treatment was withdrawn (1 day), monocytic cells were once again detected. It can be concluded that OPL98111 eradicated the monocytic cells around the time of repeated administration and once the drug had cleared, circulating cells once again migrated to necrotic areas. It must be stressed that the mouse host is the origin of the endothelial and monocytic cells since only a tumor cell-line was used to inoculate the mice and that the cells must have been **activated, proliferating** and **migrating** in order to invade the tumor tissue.

In the 30-40 days that each xenograft experiment took to complete, no animals died and post-mortem histology did not reveal any detectable abnormalities in major organs despite receiving 2.66 and 1.66 mg/kg cumulative doses (albeit intratumorally) of OPL98111 in two experiments, respectively. Single doses were in the typical range used in animal models for other ligand-toxins. If OPL98111 had been targeting cells other the those predicted this would have surely been detected

considering the lengthy experimental period and relatively high cumulative doses employed in these experiments.

In toxicology testing, a single intraperitoneal (i.p.) dose of 5 mg/kg OPL98111 (and another chemokine-toxin OPL98110) had no apparent effect on normal mice as determined by histological examination of several organs. Further, cumulative doses of 10 mg/kg OPL98110 and also of chemokine-toxin, OPL98112 to 14 mg/kg administered i.p. had no apparent effect on normal mice as determined by histological examination of several organs. These doses are far in excess that would be used in efficacy models.

Finally, a massive dose of 5 mg/kg (at least 25 times a maximum single dose) of OPL98111 administered i.v. caused the death of mice after a 24 h period. The purpose of these experiments is to define the range of toxic doses. The pathology revealed a ruptured colon but no effect on other organs. Human colon epithelial cells express CXCR4 receptors (see, *e.g.*, Jordan (1999) *J Clin Invest 104*:1061-69). Hence, this observation is consistent with the targeting to CXCR4 receptors (the target receptor for OPL98111) present on human colon epithelial cells. The cells used for the xenograft model were human colon cells. Colon cells undergo a relatively fast turnover rate and are perpetually undergoing proliferation, are therefore "activated" cells and would be expected to exhibit the upregulated expression of certain specific chemokine receptors.

Conclusions

- There is a clear nexus between the information gleaned from the published literature on the chemokine ligand and receptor biology of different cell types, and the *in vitro* and *in vivo* data presented on chemokine-toxins described here. This includes effects on tumor, endothelial, epithelial and immune cells.
- The *in vivo* toxicology data underscores the fact that three chemokine-toxins do not interfere with normal tissues/cells at massive doses (i.e.,

they do not target normal cells but most likely only pathological activated cells). Further, these studies showed that OPL98111 does affect active target cells bearing the cognate receptors *in vivo* (having no effect on non-target tissues/cells), but only at a massive non-therapeutic dose.

B. Evidence for a nexus between chemokine-toxin *in vitro* data and *in vivo* data

The application describes the roles of leukocytes in the underlying pathology of several diseases and provides a therapeutic strategy based on these roles and the observations and conclusions by myself and co-inventor that the functions of various leukocytes subtypes are tightly regulated by the chemokine family of ligands and cognate receptors, and that the temporal and spatial expression of these receptors on specific leukocyte subtypes in specific inflammatory tissues can be harnessed as a therapeutic modality. The therapeutic strategy claimed in this application provides a means to interfere with the chemokine system in a specific manner in order to treat various diseases that share a common underlying pathology. Eradication of specific "pathological" leukocyte subtypes is an optimal route for treatment.

The data described above, adequately demonstrates that conjugates prepared as taught in the application have the predicted targeting profile based upon the selected chemokine targeting agent and that such targeting is specific to the receptor. The conjugates perform as taught in the application; they target the intended cells and exhibit cytotoxic activity to such cells. This is demonstrated using well-recognized *in vitro* assays and a recognized *in vivo* model. The data indicate that the chemokine-toxin conjugates target cells with specificity and a predictably.

As discussed above, it is well established in the literature that the CXCR4 receptors targeted by OPL98111 are expressed on **activated** endothelial and immune cells (as well as the HT29 colon tumor cell line used). Based upon this and the

disclosure of the application, OPL98111 is predicted to eradicate **activated**, **proliferating** and **migrating** endothelial and monocytic cells (i.e., monocytes and macrophages). As discussed above, this is demonstrated in the xenograft model.

The application teaches that specific disruptions of the chemokine system are the hallmark of certain diseases. This finding has since been independently corroborated by countless investigators and has been the focus of several reviews (see, *e.g.*, Gerard *et al.* (2001) *Nat Immunol* 2:108-15).

As described in the prior DECLARATION, the large number of ligands and receptors for the conjugates provided in the above-captioned application make it possible to choose a suitable combination of targeting agent and receptors of a desired distribution and expose target cells with a high degree of selectivity. Disease-related chemokine receptor upregulation increases the likelihood of successful chemokine receptor-targeting conjugate target cell interactions, which, as borne out by the experiments herein, should occur at relatively low concentrations of the drug. Furthermore, the exact chemokine receptor targeting agent can be selected to suit the stage and severity of the disease. The *in vitro* and *in vivo* results demonstrate that the methods and conjugates in this application provide a means to exploit the dynamic nature of chemokine receptor distribution and upregulation that are the hallmark of pathophysiological inflammatory conditions.

As taught in the application, these agents also take treatment of disease to another level in that the chemokine-toxin selected will be defined by the leukocyte population(s) at a given stage in the pathology of the condition. For example, in the very early stages of traumatic injury to the CNS, microglia mediate inflammation and they can be targeted with OPL98110. If diagnostic testing indicates that inflammation has progressed to the point where infiltrating macrophages and neutrophils are present at the site of injury, it would be appropriate to supplement treatment with a neutrophil selective agent like OPL00202 (an IL-8-toxin conjugate). If the patient presents with still later stages of inflammation where T-lymphocytes

will be a component then and a broader acting chemokine-toxin like OPL98101 or OPL00203 would be appropriate. In arthritis, it is not just a question of the stage of disease but also subtle differences in the pathology the this disease can manifest. Macrophages appear to upregulate their production of IL-8 in the active phase of RA. This indicates that chemokine-toxin conjugates that target macrophages at the early stages, and those that target neutrophils at a later stage, would be of particular use. The production of IL-8 is low in fibrotic synovitis associated with some cases of RA, suggesting that the infiltrates do not always contain large numbers of neutrophils. Th-1 cells are selectively recruited to the joints of children with juvenile idiopathic arthritis. These cells have upregulated CXCR3 and CCR5 receptors making them good targets for other conjugates that contain chemokine receptor targeting agents that bind to those receptors.

C. The chemokine superfamily of ligands and receptors are structurally and functionally distinct from those of the cytokine family.

Although chemokines are classified as cytokines, they are a distinct class of proteins. This classification is more historical than actual. When new proteins are discovered they are named for example, after their apparent activity or their cellular source. Thus the early cytokines were thought to be hormones or were called growth factors. Because cytokines share many properties with hormones and growth factors, the distinction has been and still is a grey area. For example, in a review article (see, Wells *et al.* (1996) *Ann Rev Biochem 65*:609-34) the phrase "hematopoietic hormones/cytokines" is used (a reference to the similarity of biological activities with the various colony-stimulating factors) to describe cytokines. Some cytokine activities originally were isolated from lymphocytes and monocytic cells and were termed lymphokines and monokines, respectively. When it was realized that these molecules represent a broad spectrum of activities and were derived from numerous cell types the term "cytokine" was coined (see *e.g.*, www.umdnj.edu/pathnweb/genpath/lec_1/lec_1.htm).

Cytokines (12-40 kDa proteins) include not only interferons (IFNs), tumor necrosis factors (TNFs) and interleukins (so-called because their activity includes communication between leukocytes), but also the hematopoietic growth factors, growth hormone, ciliary neurotrophic factor and others (see, *e.g.*, Touw *et al.* (2000) *Mol Cell Endocrinol 160*:1-9). These cytokines regulate the proliferation and differentiation of a many different cell types via structurally homologous class I cytokine receptors. The Class I receptors are usually composed of two polypeptide chains, an α ligand-specific subunit and a β signal transducing subunit. This class of receptors can be subdivided on the basis of an identical α subunit and the utility of a third subunit. The interferons act via a structurally distinct set of three (α, β, and γ) Class II receptors (see, *e.g.*, Touw *et al.* (2000) *Mol Cell Endocrinol 160*:1-9). There is now an emerging family of distinct TNF receptors (Baud *et al.* (2001) *Trends Cell Biol 11*:372-7).

Cytokine receptors usually signal via the JAK/STAT intracellular signal pathway. Significantly, none of the cytokines that bind with these receptors, including IL-2, bind to any of the structurally distinct chemokine receptors (described below) and no chemokine ligand binds to any of the above described cytokine receptors. Finally, unlike chemokine receptors, none of the cytokine receptors act as co-receptors for HIV infectivity.

Chemokine Ligands and Receptors

Several unspecific leukocyte chemoattractants including the bacterial product N-formyl-methyionyl-leucyl-phenalanine, leukotrienes and complement factors were identified decades ago (see, *e.g.*, Proudfoot *et al.* (2000) *Immunol Rev 177* 246-56; Huang *et al.* (2000) *Immunol Rev 177*:52-67). Only 15 years ago, the first specific leukocyte subtype-selective chemoattractant, interleukin-8 (IL-8), was discovered. Because of its specificity and other actions on leukocytes IL-8 was not classified with the above unspecific chemoattractants but as another cytokine (interleukin). This discovery led to the search for other specific chemoattractants and it was not

until the mid nineties that it was realized that these molecules constituted a superfamily of related proteins. The distinct superfamily now includes over 50 small (8-14 kDa) chemokine ligands and approximately 20 cognate receptors, and others will be described. A new nomenclature system for the chemokine superfamily has been adopted and IL-8 has been reclassified and renamed CXCL8 (see, *e.g.*, Murphy *et al.* (2000) *Pharmacol Rev 52*:145-76).

As described in the application, chemokine superfamily is currently divided into four sub-groups (α, β, γ and δ or CXC, CC, C, CX3C, respectively) based on, the position of up to four conserved cysteine residues in the primary sequences (see, *e.g.*, Murphy *et al* (2000) *Pharmacol Rev 52*:145-76). The CXC (α) family has an intervening amino acid between the first two cysteines whereas the CC (β) chemokines do not. The C (γ) chemokines only have the second and fourth conserved cysteine residues and the more recent CX3C (δ) family members have three intervening amino acids between the first two conserved cysteines. The CX3C chemokine fractalkine is different again in that it exists in soluble and membrane bound forms. The CXC chemokines can be further divided by structure into ELR+ and ELR- molecules based on the presence or absence of tripeptide motif at the N-terminal. Chemokines exert their effects through their interaction with seven transmembrane domain receptors with (25-80% amino-acid homology) coupled to G-proteins and GPCRs. The N-terminal portion of chemokine receptors is key to determining ligand binding specificity. Unlike cytokine receptors, these GPCrs signal via the phosphoinositide (calcium mobilizing) MAP kinase intracellular signal pathways (see, *e.g.*, Thelen (2001) *Nat Immunol 2*:129-34). Finally, as mentioned above, several chemokine receptors act as co-receptors for HIV infectivity.

Although cytokines and chemokine subfamilies play major roles in the functioning of the immune system, there are major differences. Cytokine receptors are widely distributed on a various cell types and expressed in health and disease, accounting for the pleiotropic effect of their ligands. Constitutive (low) expression

of chemokines ligands and receptors are responsible for homeostatic immune system functions. In contrast, inducible chemokine ligands and receptors are expressed at low levels or not at all and are upregulated under stimulation by inflammatory stimuli including pathogens, trauma and several pro-inflammatory cytokines including, Il-1, IL-2, IFN and TNF-α (Proudfoot *et al* (2000) *Immunol Rev 177* 246-56; Huang *et al.* (2000) *Immunol Rev 177*:52-67; Gerard *et al.* (2001) *Nat Immunol 2*:108-15; Moser *et al.* (2001) *Nat Immunol 2*:123-128). Thus, cytokines can be viewed as working at a higher level of the pyramid than chemokines.

The main role of inducible or inflammatory chemokines is the recruitment of specific subtypes of leukocytes to the site of inflammation along a chemokine gradient with the induction of specific adhesion molecules (on endothelial cells and immune cells) to aid this process. The expression and functions of chemokines and their receptors are finely regulated *in vivo*. The ligands and their receptors constitute a significant locus of regulation, and, both are significantly elevated in response to pro-inflammatory stimuli. The distribution of receptors across the different cells represents another means of regulation. Each cell type has a chemokine receptor profile that is akin to a fingerprint that can be tissue, disease, and time dependent. For example, cells of monocytic lineage tend to be associated with CXCR4 and CCR2, 3 and 5 receptors, eosinophils with CCR1 and 3, neutrophils with CXCR2 and CCR1, Th1 cells with CXCR3 and CCR5, and Th2 cells with CCR3, 4 and 8, receptors. Finally, specific ligand-receptor interactions initiate specific signaling mechanisms that lead to unique cellular responses (McDonald *et al.* (2001) *IDrugs 4*:427-442; Proudfoot *et al* (2000) *Immunol Rev 177* 246-56; Huang *et al.* (2000) *Immunol Rev 177*:52-67; Gerard *et al.* (2001) *Nat Immunol 2*:108-15; Moser *et al.* (2001) *Nat Immunol 2*:123-128).

The superfamily of chemokines and cognate receptors are the principal factors that define the specific nature of the inflammatory infiltrates and this has been observed in several human diseases and animal model pathologies. Evidence comes

from examination of diseased human and animal tissues, the use of knock-out mice, transgenic mice, antibodies to ligands and receptors and chemokine receptor antagonists. Chemokines and their receptors have been implicated in the activation, migration and proliferation of different leukocyte sub-types in human diseases (of almost every organ system) including atherosclerosis, arthritis, asthma, HIV/AIDS, kidney disease, multiple sclerosis, transplant rejection and numerous cancers (see references immediately above). The above observations, the nature of the specificity of chemokine ligand and receptor expression in various diseases and the structural nature of the receptors has made them prime targets for therapeutic intervention.

D. The teachings of Volk *et al.* and also the efficacy of different immune cell-depleting agents

Volk *et al.*

In the rejection under 35 U.S.C. §112, first paragraph, of the Detailed Action (page 3), the Examiner cites results described by Volk *et al* of the unpredictability of *in vitro* versus the *in vivo* activity of IL-2-PE40. This is cited as evidence that this unpredictability would be expected of chemokine-toxins since "chemokines are considered cytokines." This is not a correct inference.

Volk *et al.* notes that *in vivo* data had previously shown that although IL-2-PE40 exhibits immunosuppressive efficacy, observations suggested that the humoral response was not inhibited (abstract, p 2497). The Volk *et al.* studies were designed to address this anomaly. The studies show the dichotomous effects of the fusion protein *in vitro* and *in vivo* and thus **provide a nexus** for the activity of this particular fusion in the two paradigms. The studies establish that the fusion initially stimulates (or activates) II-2 receptor bearing cells before killing them.

The undesirable humoral response reported by these investigators is understandable. IL-2 binds high and low affinity receptors and presumably the latter are expressed on B-cells. This explains the transient stimulation of the B-cells and antibody production upon stimulation with the IL-2 moiety. The entire molecule

would have been internalized slowly thus giving a delayed toxic effect on these cells. In conclusion, this study merely dissects the mechanism of action of an IL-2 directed toxin which is consistent with the cell biology of the actions of this molecule and its cognate receptors. It does not bear relevance to the biological actions of any other cytokine (e.g., TNF) or any chemokine because the biological actions are so different.

The rejected claims in the instant application are directed to methods of targeted delivery of an agent into cell that express chemokine receptors (*i.e.*, independent claim 35) and a method of inhibiting proliferation, migration and activation of cells bearing chemokine receptors (*i.e.*, independent claim 38). As pointed out above, none of the classic cytokine ligands including IL-2, binds with any of the structurally distinct chemokine family of receptors and no chemokine ligand binds to any of the Class I or II cytokine receptors. Thus neither IL-2 nor any other cytokine (in the classic sense) ligand is capable of targeting the delivery of any agent via any given chemokine receptor and hence would not be able to affect proliferation, migration or activation of cells via a chemokine receptor.

The Volk *et al.* studies do not disclose or suggest the use of chemokine-toxins for treating leukocyte-mediated diseases and do not teach any unpredictability of *ex vivo* and *in vivo* responses (mechanisms of action) of the unique tightly regulated chemokine ligand-receptor superfamily. Unlike chemokine receptors, the cytokine receptors are widely distributed and not under exquisite and tight regulation, and this accounts for the pleiotropic nature of cytokine ligands.

In the section above describing differences between classic cytokines and the super family of chemokines makes it very clear that these families carry out different immune functions and at different levels of regulation. Hence chemokines cannot be considered classic cytokines and *vice versa*.

Furthermore, it is not reasonable to compare the actions of two families of receptors and ligands where the structures, functions and cell biology are unrelated

and highly distinct. Volk *et al.* does not suggest selection of a different "cytokine", since the negative data would have dissuaded further experimentation. In addition, it would have not been clear which ligand would have made a suitable substitute for IL-2 be it cytokine or chemokine given the diverse biological actions . Finally, the distinction between using an inducible (inflammatory) versus constitutive (homeostatic) chemokine ligand would not have been apparent.

Numerous ligand-toxin fusion proteins, including in IL-2 fusion construct, have entered clinical trials and several have been FDA approved. This includes the IL-2-diphtheria toxin fusion, "Ontak", which is essentially the Volk *et al.* conjugate. The widespread nature of the distribution of target receptors on normal and diseased cells for numerous antibodies (on immunotoxins) and cytokines is well understood. This accounts for the toxicity profiles noted for such compounds including Ontak. Investigators have used this knowledge to implement a limited dosing regime of such (potential) therapeutics. The human dose for Ontak is limited to 63 μg/kg for lymphoma and only few doses are administered (see, *e.g.*, insert sheet at www.ligand.com). It is interesting to note that, if dosing regimes are restricted, even potentially toxic molecules can get FDA approval.

Efficacy of different immune cell-depleting agents

In the Office Action, the Examiner states that Bexxar and Genimmune, which were cited by the applicants to demonstrate "operativeness" and to "evidence" confirmation of what is taught in the instant application, have not received FDA approval, concludes that the effectiveness of these compounds and their use in treating immune diseases has not yet been established. These compounds, however, are in clinical trials, (the former to Phase III) and thus have shown efficacy not only in animal models but also in humans. Bexxar is awaiting FDA approval.

In addition, two other fusion proteins have been approved by the FDA namely Zevalin for lymphoma (from IDEC Pharamceuticals) and Myelotarg (AHP) for leukemia. Ontak the IL-2 fusion, discussed above, from Ligand Pharmaceuticals

targets T-cells, is (FDA) approved for Lymphoma and is in Phase II trials for the T-cell mediated condition of Psoriasis. Novantrone is a DNA intercalating chemical that has FDA approval for the treatment of progressive multiple sclerosis. This agent is essentially an anticancer drug and is cytocidal to proliferating macrophages and T-cells which are the recognized cells underlying the pathology of the disease (insert sheet from www.amgen.com). Colchicine is a plant derivative approved by the FDA for gout and arthritic gout. The anti-inflammatory effects of this molecule are thought to be by inhibiting the proliferation of immune cells by binding to intracellular microtubules that are essential for meiosis and mitosis. Finally, many antibodies are under preclinical and clinical development for a wide array of immune cell mediated diseases. For example, anti-CD147 monoclonal antibody (ABX-CBL) from Abgenix (www.abgenix.com) has completed a Phase II trial for acute graft-versus-host disease. The antibody kills different groups of immune cells through complement-mediated lysis.

These examples (and there are many more) demonstrate that elimination of immune cells in various diseases is a valid approach to therapy. They approach the elimination by different means and, thus, do not teach the eradication of such cells by using agents that exploit chemokine receptors or their ligands as claimed in this application. The beauty of the chemokine system that is exploited in the instantly claimed methods, is that there is a greater deal of targeting specificity and versatility. For example CCR3 expressing TH2 cells and eosinophils are implicated in the pathology of allergic asthma. The fusion with the eotaxin ligand (OPL98112) can eliminate both types of pathological cells; whereas an antibody can only eliminate on type or the other. OPL98112 exhibits no gross toxicity *in vivo* using normal animals (mentioned above).

Conclusions

- Leukocyte eradication is a viable therapeutic strategy for a wide range of diseases.

- There are numerous examples of drugs, proteins (antibodies) and ligands –toxins (fusions) that eradicate pathological immune cell populations in a wide range of diseases or disease models. These examples provide ample instruction on how to ratify the use of new proteins/drugs/antibodies/fusions including chemokine-toxins.

* * *

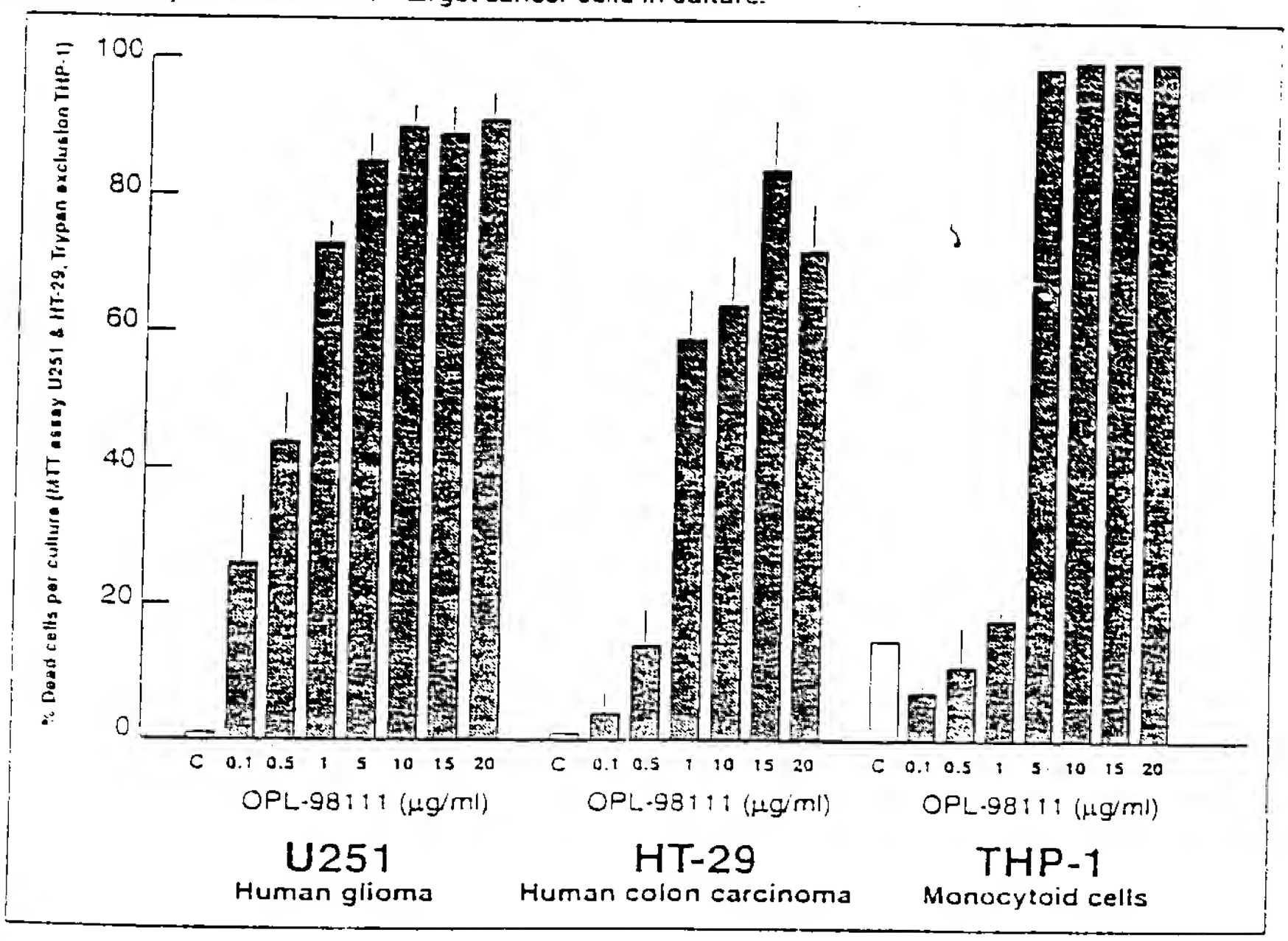
I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

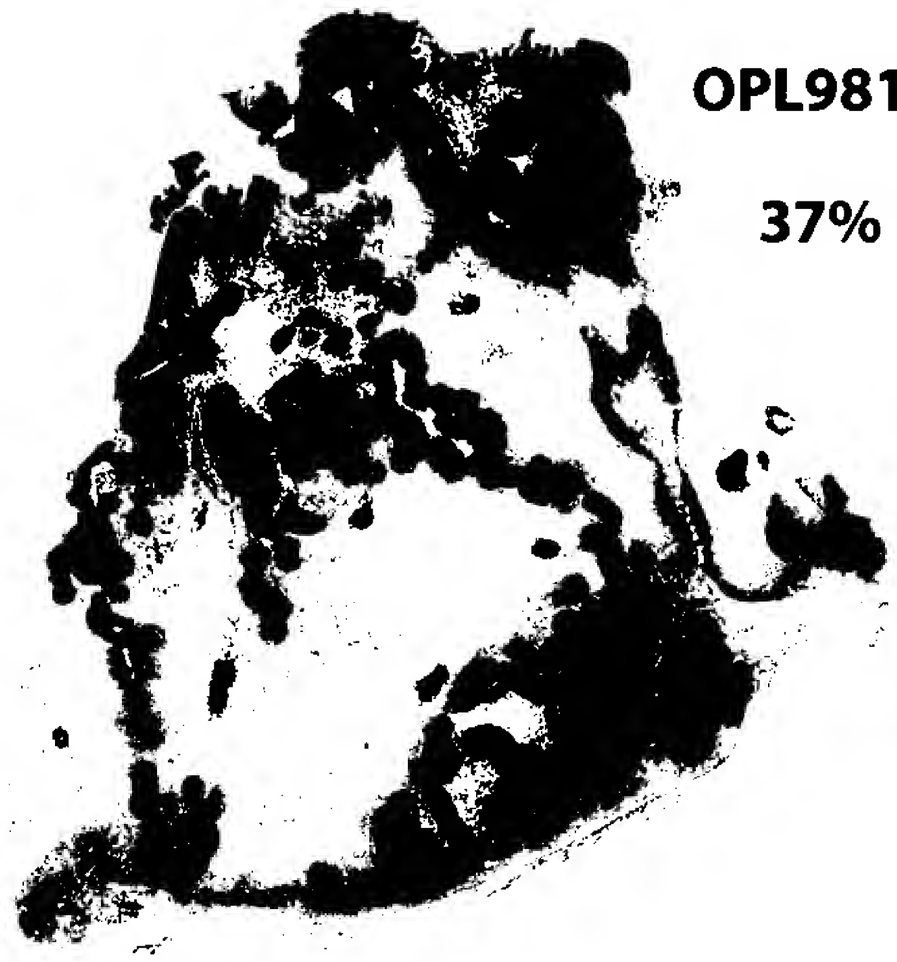
JOHN R. McDONALD

Date: 10/26/01

25020-601B

FIGURE 1 Cytotoxic activity of OPL-98111 on target cancer cells in culture.





OPL98111 Treated

37% Live Tumor

Untreated

68% Live Tumor

H&E stained cross section of HT-29 Human Colon Carcinoma tumors xenografted on the flank of SCID/CB17 Fox Chase Mice.

In these size-matched tumors the OPL98111 treated tumor exhibits far less live tumor mass (dark violet staining; 37% versus 68% in control) and a larger area of necrosis (lighter pink staining). The untreated tumor shows much greater evidence of vascularization (bright white dots) and at higher magnification, the treated tumor contained abundant monocytic cells — presumably in the process of clearing cellular debris.